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**Mitochondrial DNA sequence divergence between “Kunimasu” *Oncorhynchus kawamurae*  
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identification using multiplex haplotype-specific PCR**

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Short running head: MHS-PCR identification of *Oncorhynchus kawamurae*

Short report

12 text pages

2 figures

**Abstract** In order to develop a simplified method for discriminating between *Oncorhynchus kawamurae* (Kunimasu) and sympatric *Oncorhynchus nerka* (Himemasu), sequence variations of mitochondrial DNA control region were examined on 71 specimens of Kunimasu and 103 of Himemasu. Sequence analyses for 530 bp revealed 3 variable sites, which defined three haplotypes in Kunimasu and one in Himemasu. Based on determined nucleotide sequences, species-specific primers were designed for the multiplex haplotype-specific polymerase chain reaction, which amplified ca. 900 and 1,040 bp fragments in Kunimasu and Himemasu, respectively. This simple rapid approach can be used to distinguish between the two species without the need to sacrifice specimens.

**Key words:** *Oncorhynchus kawamurae* · Lake Saiko · Molecular identification

## Introduction

*Oncorhynchus kawamurae* Jordan and McGregor in Jordan and Hubbs 1925 (common name “Kunimasu”), formerly restricted to Lake Tazawa, Akita Prefecture, the only known habitat of the species, had long been believed to have become extinct since the late 1930’s. Recently, however, Nakabo et al. (2011) discovered Kunimasu in Lake Saiko, Yamanashi Prefecture, one of the lakes to which eyed eggs of Kunimasu had been introduced from Lake Tazawa in 1935.

Stock assessment and intensive conservation efforts for Kunimasu are urgently needed, due to its present restricted distribution, i.e., Lake Saiko only. Although Kunimasu is known to have distinct morphological and ecological characteristics, having few pyloric caeca, and occurring and spawning on the deep bottom (Akitaken Suisanshikenjo 1915; Tanaka 1911; Okuyama 1939; Oshima 1941; Sugiyama 2000), both being unique within *Oncorhynchus*, comprehensive biological studies had at no time been conducted on Kunimasu in Lake Tazawa before its extinction. However, the discovery of Kunimasu in Lake Saiko has provided an unexpected opportunity to learn more of the morphological characteristics and life history of the species, especially with regard to spawning ecology and adaptations to its deeper, cooler water environment.

Kunimasu has been considered to have a close relationship with Kokanee, the land-locked sockeye salmon *Oncorhynchus nerka* (Japanese common name “Himemasu”) (Hosoya 2002; Eschmeyer 2012), sometimes having been treated as a subspecies or variation of the latter. Because Himemasu has been vigorously introduced to Lake Saiko over many years, both Kunimasu and Himemasu now occur sympatrically in the lake. However, the two species are clearly reproductively isolated (Nakabo et al. 2012; Muto et al. 2012), and are distinguishable by the numbers of pyloric caeca and gill rakers, in addition to spawning season and body coloration at such times (Jordan and Hubbs 1925; Akitaken Suisanshikenjo 1931; Oshima 1941; Hikita 1962; Sugiyama 2000). Notwithstanding, however, younger (immature)

and non-spawning adult fish show few differences in external morphology or coloration (Muto et al. 2012), resulting in difficulties in identification of the two species. Furthermore, it has been suggested that there is little difference in microhabitat or swimming depth between Kunimasu and Himemasu in Lake Saiko (Muto et al. 2012), unlike the situation in Lake Tazawa (Sugiyama 2000).

Detailed biological or ecological studies on Kunimasu in Lake Saiko would clearly be enhanced by a practical and reliable means of distinguishing between the former and Himemasu throughout all life history stages, from eggs to post-spawning carcasses. Morphological identification techniques, such as counting the numbers of pyloric caeca and gill rakers, are both time-consuming and labor intensive, apart from being inapplicable to eggs or juveniles. In addition, an identification method that does not sacrifice specimens is highly desirable, due to the apparently limited number of living individuals. Although microsatellite DNA analysis can discriminate precisely between Kunimasu and Himemasu without harming specimens, it is time-consuming and expensive, due to the need for genotyping at several loci (> 5), owing to the apparent absence of a diagnostic allele for Kunimasu (Muto et al. 2012). In addition, microsatellite DNA analysis usually requires an expensive automated fluorescent DNA sequencer, which makes general application of the method difficult.

This study aimed to develop a simple, rapid, cost-saving molecular method for discriminating between Kunimasu and Himemasu in Lake Saiko. We applied multiplex haplotype-specific (MHS) PCR to mitochondrial (mt) DNA for these species, such being a single step method based on haplotype-specific variations in DNA sequences (e.g., Rocha-Olivares 1998; Durand et al. 2010; Yamanoue et al. 2010). On the premise that species-specific DNA sequence variations exist, this method was expected to be labor, cost and time-saving, and applicable even to small juveniles, yet yielding reliable, sensitive and highly reproducible identifications (reviewed in Teletchea 2009), all the while without the need to harm specimens.

## Materials and methods

*Specimens.* A total of 144 specimens, including 71 Kunimasu and 73 Himemasu, collected from Lake Saiko in 2010 and 2011 by gill net and angling, or the collection of carcasses (“Uki-yo”) washed ashore after spawning, plus 30 specimens of indigenous Himemasu from Lake Akan (but see Kaeriyama 1993), in which Himemasu is not sympatric with Kunimasu, were utilized in the study. The same specimens were previously used for microsatellite DNA analyses by Muto et al. (2012). The specimens were unambiguously assigned to either Kunimasu or Himemasu, using Bayesian clustering, and deposited in the Kyoto University Museum (FAKU) or the Yamanashi Prefectural Fisheries Technology Center (YFTC).

*DNA extraction, PCR amplification and sequencing.* Total genomic DNA was extracted from white muscle preserved in 99 % ethanol using the DNeasy Tissue Kit (Qiagen, Germany) or the Wizard Genomic DNA Purification Kit (Promega, USA), both following the manufacturers’ protocols. The control region of mtDNA was amplified by PCR, using the forward primer tRNA<sup>thr</sup>-2 5’-TCT TGT AAT CCG GAA GTC GGA-3’ and reverse primer tRNA<sup>phe</sup>-2 5’-AAC AGC TTC AGT GTT ATG CT-3’ (Sato et al. 2001). PCR was conducted using the Takara EX *Taq* polymerase kit (Takara Bio Inc., Shiga, Japan) under the following temperature profile: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products were purified with ExoSAP-IT (USB, Co., OH, USA) and directly sequenced using the BigDye 1.1 Terminator sequencing kit (Applied Biosystems Inc., CA, USA) following the manufacturer’s protocol, with the primer tRNA<sup>thr</sup>-3 5’-GGT TAA AAC CCT CCC TAG TG-3’ (Sato et al. 2001). Alignments were checked by eye with the help of DNASIS software (Hitachi Software Engineering Co.,

Kanagawa, Japan).

*Primer design for MHS-PCR.* On the basis of sequence variations among Kunimasu and Himemasu, species-specific forward primers were designed to anneal to sequences with a diagnostic nucleotide for each species on the 3'-end, taking intra-specific sequence variations into account, and to amplify different-sized PCR products. To enhance the specificity of primer annealing, the third nucleotide from the 3'-end of each primer was designed to be mismatched, as noted in Sommer et al. (1992) and Watanabe and Umetsu (2007). The sequences of the designed primers were as follows: Ok-spL (Kunimasu specific primer) 5'-ATT ATC AAC ATA CRG TGG TGTC-3'), On-spL (Himemasu specific primer) 5'-TAA ACT ACC CTC TGA CGG CTAC-3'). Via MHS-PCR using these species-specific forward primers and the common reverse primer tRNAphe-2 (see above), a species-specific fragment of ca. 900 base pairs (bp) in Kunimasu and ca. 1040 bp in Himemasu were predicted to be amplified. For internal positive control of the PCR conditions, a pair of primers expected to commonly anneal to either of the two species, L-M-12S; 5'-ACA AAC TGG GAT TAG ATA CCC CAC TAT GCC-3' and H-A-16S; 5'-GTT TTT GGT AAA CAC GCG AGG CTT ATG TTT GCC GAG-3' (Matsui et al. 2012), which are complements of the primers of Kawaguchi et al. (2001), producing a ca. 1,500 bp amplicon, were also included in MHS-PCR. Thus, for either of the two species, two different-sized fragments (a species-specific amplicon and internal positive control) could be expected to be amplified.

*MHS-PCR amplification.* MHS-PCR was performed on some representative specimens selected so as to involve all haplotypes of the two species found in this study, using five primers [two species-specific forward primers (Ok-spL and On-spL), a reverse primer common to the two species (tRNAphe-2), and a pair of primers for internal positive control (L-M-12S and H-A-16S)]. The PCR reaction was conducted using the Takara *Taq* Hot Start Version polymerase kit (Takara Bio Inc., Shiga, Japan), with a reaction mixture containing 1.0 µl of 10X provided buffer, 1.0 µl of provided dNTPs mixture, 1.0 µl each of five primers, 0.06

μl of Takara *Taq* Hot Start Version polymerase and 0.40 μl of extracted total genomic DNA as template, with the total volume brought up to 10 μl with sterilized water. The PCR temperature profile was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 15 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products, together with a DNA size standard, were electrophoresed on a 1.5 % agarose gel in TAE buffer and visualized with ethidium bromide stain.

## Results

Nucleotide sequences were obtained for 530 bp containing the part of the mitochondrial proline transfer RNA gene and the 5' end of the control region for both Kunimasu and Himemasu. Three variable sites were found among 174 specimens, with two transitions, one transversion and no indels, thereby yielding three and one haplotypes from Kunimasu and Himemasu, respectively. No haplotypes were shared between the two species. The names of haplotypes and their frequency in Kunimasu were as follows: K-01 (85.9 %), K-10 (11.3 %) and K-29 (2.8 %). A representative haplotype of Kunimasu (K-01) was deposited in the DNA Data Bank of Japan (DDBJ), with the accession number AB774473. The uncorrected pairwise sequence divergence between species was 0.4–0.6 %. Two diagnostic nucleotide sites were found, making it possible to design species-specific primers for each species and employ the MHS-PCR method for discriminating between the two species. Inter- and intraspecific variations of aligned sequences at the annealing sites of the designed species-specific primers are shown in Fig. 1, and the MHS-PCR results for each of three specimens each of Kunimasu and Himemasu in Fig. 2. As predicted, each Kunimasu (lanes 1–3 in Fig. 2) and Himemasu individual (lanes 4–6 in Fig. 2) showed a species-specific fragment of ca. 900 and ca. 1,040 bp,



respectively, plus a common fragment of ca. 1,500 bp.

## Discussion

Kunimasu has been considered to have a close affinity with Himemasu (*Oncorhynchus nerka*, otherwise referred to as kokanee or anadromous sockeye salmon) (Sugiyama 2000), although their exact evolutionary relationship remained unclear, because only 17 formalin-fixed specimens of Kunimasu from the species' original habitat, Lake Tazawa (including the holotype, paratype and nontype specimens), were known prior to its rediscovery in Lake Saiko. In addition, an attempt to extract DNA from formalin-fixed Kunimasu specimens had resulted in failure (Sugiyama 2000). Recently, Muto et al. (2012) suggested that Kunimasu represented one of a number of populations of kokanee, based on comparisons of genetic distances obtained from microsatellite DNA data from Kunimasu, Himemasu and populations of kokanee or sockeye salmon from the Pacific rim. Mitochondrial DNA sequence information in the present study indicated that the relationship of Kunimasu with Himemasu was the closest amongst species of *Oncorhynchus*, the uncorrected pairwise sequence divergence between the former being only 0.4–0.6 %, whereas those between Kunimasu and other *Oncorhynchus* species were > 5 % (calculated from DDBJ/EMBL/GenBank sequence data: Accession nos. AB039892; *Oncorhynchus keta*, NC010959; *O. gorbuscha*, NC009263; *O. kisutch*, NC002980; *O. tshawytscha*, DQ864465; *O. masou*, NC001717; *O. mykiss*).

The result of MHS-PCR indicated that Kunimasu from Lake Saiko could be clearly distinguished from sympatric Himemasu, by differences in the sizes of PCR products. In this method, *Oncorhynchus masou*, another *Oncorhynchus* species inhabiting in Lake Saiko, yielded neither of the two species-specific fragments but only the internal positive control fragment (the result not shown). This method is advantageous in time or cost than other molecular

identification methods such as PCR restriction fragment length polymorphism (PCR-RFLP) (e.g., Larmuseau et al. 2008) or microsatellite DNA analysis (e.g., Madsen et al. 2009). The method is clearly applicable to field research, including ecological, stock management and enhancement, and conservation studies, without any need for further appraisal. It is important to note, however, that the method is based upon interspecific differences in mitochondrial (mt)DNA, which is a maternally inherited non-recombinant. Accordingly, identifications using mtDNA may not always be appropriate if introgressive hybridization between species is likely. In Lake Saiko, no common haplotype was found between Kunimasu and Himemasu, genetically identified from biparentally-inherited microsatellite DNA analyses, in this study. This suggests that any effects of introgression would be very minor, if present at all, in Lake Saiko. Notwithstanding, Muto et al. (2012), reported two specimens, identified as hybrids of Kunimasu and Himemasu, the Himemasu genome being dominant, as preliminary results from Lake Motosu, Yamanashi Prefecture, to which eyed eggs of Kunimasu had also been introduced in 1935 (Sugiyama 2000). We observed that one of these specimens had a haplotype of Kunimasu and the other had a haplotype of Himemasu (data not shown). Furthermore, Yamamoto et al. (2011) and Kogura et al. (2011) reported some mtDNA sequence variation in Himemasu, although we found no polymorphism within Himemasu specimens from Lake Saiko and Lake Akan. Caution must therefore be exercised when applying MHS-PCR identification techniques for Kunimasu to specimens from lakes other than Lake Saiko.

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## Figure legends

**Fig. 1** Partial sequences of mitochondrial DNA control region and proline transfer RNA gene of Kunimasu *Oncorhynchus kawamurae* and Himemasu *O. nerka*. Annealing locations of species-specific primers designed in this study indicated by arrows. Dots indicate nucleotides identical to those of haplotype K-01. Nucleotide positions numbered from the 5'-end of determined sequences. Shaded areas represent target sequences of the species-specific primers. All haplotypes found in this study are presented

**Fig. 2** An ethidium bromide-stained 1.5 % agarose gel with TAE buffer after electrophoresis of the MHS-PCR products. Lanes 1–3: Kunimasu *Oncorhynchus kawamurae* (lane1–3 correspond to K-01, K-10 and K-29 haplotypes, respectively), lanes 4–6: Himemasu *O. nerka*, lane 7: 100-bp DNA ladder size marker (Takara Bio Inc., Shiga, Japan). K, H and IC represent species-specific fragments for Kunimasu (ca. 900 bp), Himemasu (ca. 1,040 bp) and internal positive control, respectively

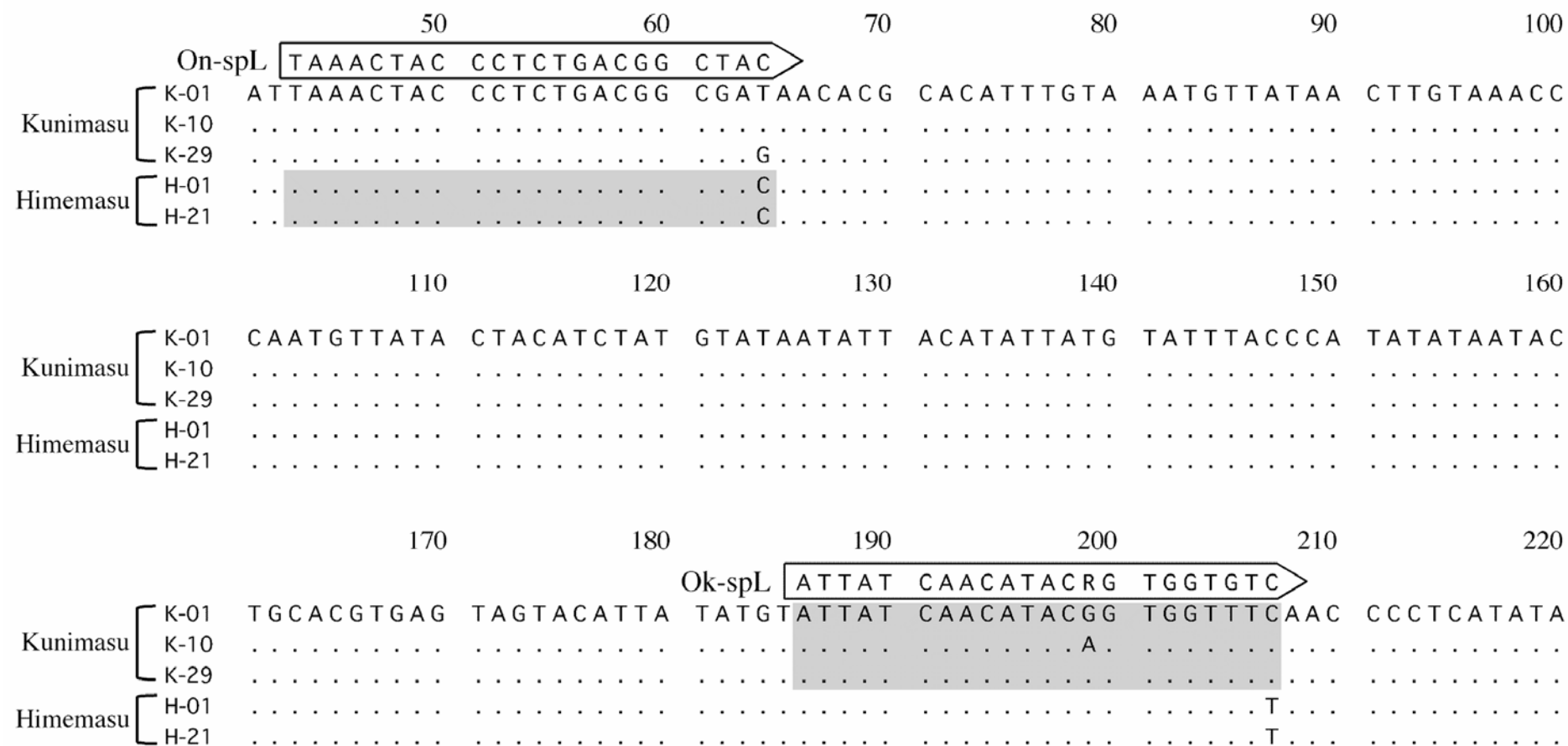


Fig. 1

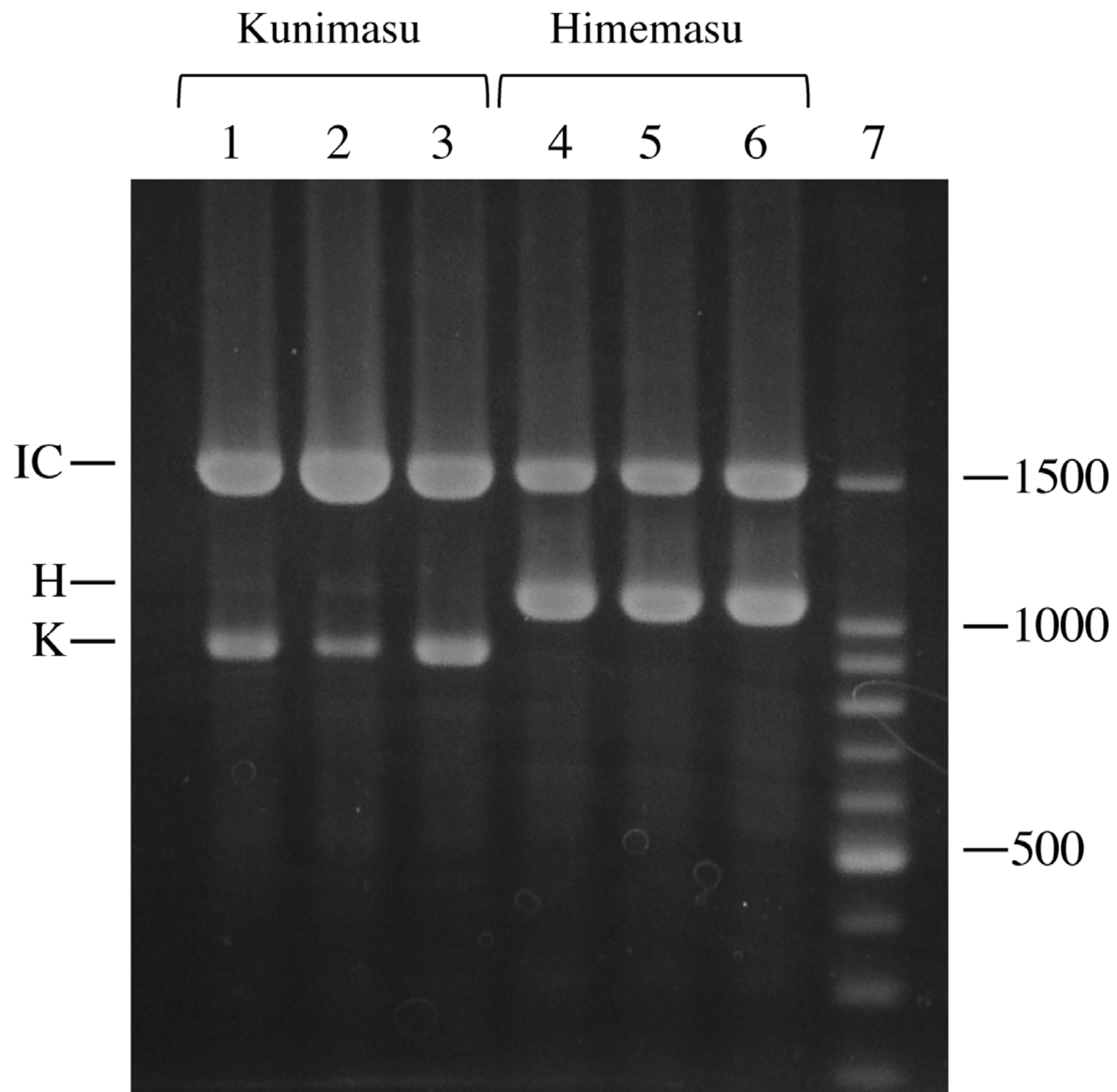


Fig. 2